Hydrolyzable Hemoglobin Adducts of Polyfunctional Monocyclic *N*-Substituted Arenes as Dosimeters of Exposure and Markers of Metabolism

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Hemoglobin adducts of 10 polyfunctional amino- and nitro-substituted benzenes and toluenes were analyzed: 2,4,6-trinitrotoluene, 2,4- and 2,6-dinitrotoluene, 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, 2,4- and 2,6-diaminotoluene, 1,3-dinitrobenzene, 1-amino-3-nitrobenzene, and 1,3-diaminobenzene. A single dose (0.5 mmole/kg) of the test compounds was administered to female Wistar rats by gavage, and blood extracted and hemoglobin prepared after 24 hr. One or more cleavage products could be obtained in each case by hydrolyzing hemoglobin (Hb). Hemoglobin binding indices (HBI: binding [mmole/mole Hb]/dose [mmole/kg]) and the ratios of hydrolyzable adducts were determined. The HBI ranged between <0.02 and 69.0. The results indicate the *in vivo* formation of several, covalently bound, hydrolyzable hemoglobin adducts. Conclusions on prevailing metabolic pathways can be drawn. Total binding of several compounds seems sufficient for biomonitoring of human blood samples. These chemicals are considered representative for environmental contamination with explosives of this type, and we propose their Hb adducts be used as dosimeters for human exposure to these suspected carcinogens. — Environ Health Perspect 102(Suppl 6):43–45 (1994)

Key words: biomonitoring, explosives, hemoglobin adducts, aminoarenes, nitroarenes

Introduction

Polyfunctional, monocyclic amino- and nitroarenes are widespread intermediates in chemical industries. They have recently attracted attention as soil contaminants in areas of former production and use of explosives (*1*). Many of them were found mutagenic and carcinogenic in appropriate test systems, and may also be hazardous for humans if absorbed and metabolically activated (*2*).

In vivo metabolism comprises reduction of nitro groups and oxidation of amino groups, resulting in N-nitroso intermediates that bind to the SH-group of cysteine in hemoglobin (Figure 1) (*3*). The resulting sulfinamide can be hydrolyzed, which yields one or more cleavage products. These can be extracted and analyzed with high sensitivity by HPLC/ECD. In case of polyfunctional derivatives, multiple cleavage products can result, depending on the metabolic reactions that have occurred with the nonbinding substituent. The measurement of hemoglobin adducts has been proposed for biomonitoring of human blood samples (*4–6*). As a basis for such an application, hemoglobin binding was studied in animal experiments and the cleavage products were identified.

Materials and Methods

The test compounds 2,4-diaminotoluene, 2,4- and 2,6-dinitrotoluene, 1,3-diaminobenzene, 1,3-dinitrobenzene, and 1-amino-3-nitrobenzene were purchased from Merck; 2,6-diaminotoluene from Fluka; 2,4,6-trinitrotoluene from Promochem; and 2-amino-6-nitrotoluene, 2-amino-4-nitrotoluene, and 2-nitro-4-aminotoluene from Aldrich. Samples of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene were kindly provided by Dr. Kaiser from the Bundesinstitut für chemisch-technische Untersuchungen in Swistal, Germany. Acetylated aminoarenes and nitroarenes, used as reference compounds and as external standards, were synthesized by standard procedures. The purity of all compounds was checked by HPLC/UV and GC–MS, and chemicals were recrystalized, if necessary.

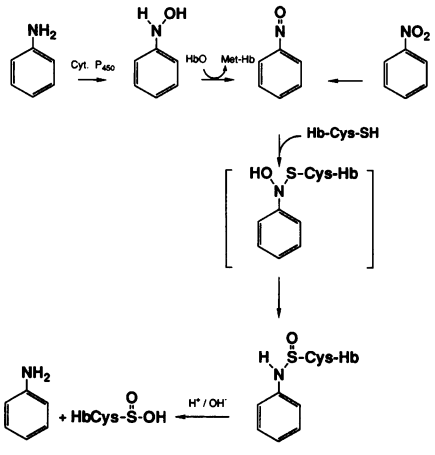

Figure 1. Formation of cysteine-S-hemoglobin adducts following the generation of *N*-nitroso intermediates after reduction of nitrogroups or oxidation of amino groups or both.

Female Wistar rats (200–250 g), obtained from the Zentralinstitut für Versuchstierkunde (Hannover, Germany) had free access to food (Altromin 1324) and water. The test compounds (0.5 mmole/kg) were dissolved in 200 to 250 µl 1,2-propanediol, 1,2-propanediol and ethanol (70/30), or tricaprylin and administered by gavage to three animals per compound.

Blood was obtained after 24 hr by heart puncture under ether anesthesia with a heparinized syringe. Blood was centrifuged at 2000*g* for 5 min, and red blood cells were washed three times with 0.9% NaCl solution. After lysis of the cells in 4 vol EDTA (10^{-4}M, pH 7.5), and centrifugation at 4000*g* for 5 min, hemoglobin was precipitated from the supernatant by dropwise addition of 4 vol ethanol while stirring. The precipitate was then centrifuged for 5 min at

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Table 1. Type of substitution of the test compounds, total amount of hydrolyzable adducts, hemoglobin-binding indices (HBI).[a]

Functional group	Toluenes		Benzenes	
	Substitution	HBI	Substitution	HBI
Trinitro-	2,4,6	6.0 ± 2.0		
Dinitro-	2,6	1.2 ± 0.4		
	2,4	0.7 ± 0.4	1,3[b]	69.0 ± 40.8
Aminonitro-	4,2	0.1 ± 0.01		
	2,4	1.0 ± 0.4	1,3	3.1 ± 0.9
Diamino-	2,6	0.15 ± 0.08		
	2,4	< 0.02	1,3	0.7 ± 0.13

[a] $n = 3$; mean ± SD. [b] $n = 2$.

Table 2. The test compounds and the composition of the products obtained by hydrolysis of the hemoglobin adducts.

Test compound	Cleavage product	Fraction, %
2,4,6-Trinitrotoluene	4-Amino-2,6-dinitrotoluene	100
2,6-Dinitrotoluene	2-Amino-6-nitrotoluene	84
	2,6-Diaminotoluene	16
2,4-Dinitrotoluene	2-Nitro-4-aminotoluene	100
2-Amino-4-nitrotoluene	2,4-Diaminotoluene	90
	2-Acetylamino-4-aminotoluene	10
4-Amino-2-nitrotoluene	4-Amino-2-nitrotoluene	100
2,6-Diaminotoluene	2-Acetylamino-6-aminotoluene	100
2,4-Diaminotoluene	Not detectable	
1,3-Dinitrobenzene	1-Amino-3-nitrobenzene	81
	1-Acetylamino-3-aminobenzene	13
	1,3-Diaminobenzene	6
1-Amino-3-nitrobenzene	1-Amino-3-nitrobenzene	62
	1-Acetylamino-3-aminobenzene	23
	1,3-Diaminobenzene	15
1,3-Diaminobenzene	1-Acetylamino-3-aminobenzene	90
	1,3-Diaminobenzene	10

10,000*g*. Precipitated hemoglobin was washed with ethanol and water (80/20), ethanol (96%), ethanol and ether (25/75), and ether, dried over silica in a desiccator, and stored at –18°C.

For adduct analysis, 10 to 50 mg hemoglobin was hydrolyzed using structurally similar recovery standards in a solution of 0.3 ml 0.5% SDS, 3 ml *aqua bidest*, and 0.3 ml 10 N NaOH (alkaline hydrolysis) or 3 ml 10% H_3PO_4 (acidic hydrolysis). The mixture was stirred for 1 hr at room temperature. After centrifugation and solid phase extraction (Polysorb MP-1 columns, 100 mg, Interaction Chemicals Inc., or Bond elut SCX-columns, 100 mg, Analytichem International, after acidic hydrolysis), an internal standard (Anilin, 1-amino-4-nitrobenzene) was added, and the extract was concentrated under a stream of N_2. Recovery was determined with hemoglobin from untreated control animals by addition of standard solutions, under identical conditions.

The samples were analyzed by HPLC/ECD using a Waters model 590 HPLC pump with a Rheodyne injector (model 7125). The electrochemical detector (ESA model 5100 A) was equipped with an analytical cell (model 5011), and was operated at potentials of 0.6 to 0.85 V. The following columns were used: LiChrospher 60 RP-select B, 5 µm, 250 × 4 mm (Merck); Superspher 60 RP-8, 4 µm, 250 × 4 mm (Merck); Cyclobond 1, 5 µm, 250 × 4 mm (Astec); or Zorbax ODS, 5 µm, 250 × 4 mm (Bischoff). Precolumns, 20 × 2 mm (GAT-Analysentechnik) were filled with Perisorb, 20 to 40 µm (Merck). Sodium phosphate buffers (0.02 M, pH 2.3–5.8) with varying amounts of acetonitrile and methanol were used as eluents. Chromatograms were quantified by comparison with external standard solutions (Figure 2) using an Epson AX3s computer and integration software from PE Nelson (Cupertino, CA). The total amount of hydrolyzable adducts was calculated as hemoglobin binding index (HBI: binding [mmole/mole Hb]/dose [mmole/kg]).

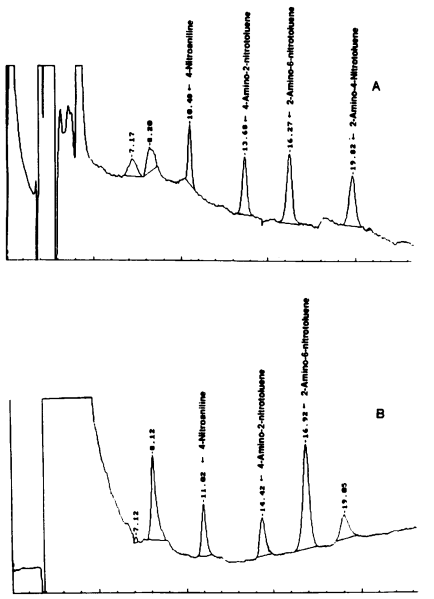

Figure 2. HPLC/ECD chromatogram of the standard mixture (*A*) and of the cleavage products extracted after hydrolysis of hemoglobin from an animal, treated with 2,4-dinitrotoluene (*B*). In this case, 4-nitroaniline and 2-amino-6-nitrotoluene were added as internal and recovery standards, respectively.

Results

After a single oral dose of the amino- and nitroarenes to female Wistar rats, all test compounds except one formed adducts that could be hydrolyzed from hemoglobin preparations under mild acidic or alkaline conditions. The exception was 2,4-diaminotoluene. The detection limit was in the range of 10 to 50 ng/g Hb for each cleavage product. Recovery of the substances was between 36 and 82%. None or extremely small amounts of the cleavage products were detected in control experiments in which hemoglobin was treated under neutral, but otherwise unchanged, conditions.

Animals treated with 1,3-dinitrobenzene developed visible met-hemoglobinemia (met-Hb) with the dose that was administered. One of them died after a few hours. These observations are in line with the known potency of this compound for met-Hb formation and its extent of hemoglobin binding. The HBIs of the two surviving animals were 98 and 40, respectively. However, the dose of 1,3-dinitrobenzene (0.5 mmole/kg) corresponded to the median lethal dose reported by Cody et al. (*7*) as 83 mg/kg (0.53 mmole/kg).

In general, toluene derivatives bind less extensively to hemoglobin than the correspondingly substituted benzenes. The total amount of hydrolyzable adducts varied

within a wide range; this is given in Table 1 as the HBI. Up to three cleavage products were detected, but one dominated in all cases. A specific pattern of hydrolyzable adducts was obtained for each chemical. The identified cleavage products and the percentage of the hydrolyzable and identified adducts are listed in Table 2. The identification is based on HPLC cochromatography with authentic reference compounds under different conditions. Major cleavage products were also analyzed by GC–MS.

Discussion

Because metabolites that bind covalently to the SH-group of cysteine in hemoglobin are nitroso derivatives (*3,8*), our results demonstrate that such metabolites are formed either by *in vivo* reduction of a nitro group or by oxidation of an amino group. The chemical structure of the parent compounds apparently influences the metabolism in each case in a specific way, and thus determines the bioavailability of the reactive intermediate. With many of the compounds tested, *N*-acetylated adducts were formed. However, the ratio of acetylated to nonacetylated cleavage products was quite variable, which again indicates specific pharmacokinetics for each substance. No hydrolyzable adduct above the detection limit was obtained with 2,4-diaminotoluene. This is probably because of more extensive *C*-oxidation, including that of the methyl group.

The total amount and pattern of cleavage products permits the following conclusions: first, reduction of nitro groups exceeds *N*-oxidation of amino groups, as indicated by adduct formation. The low levels of hemoglobin binding by diamino-substituted compounds may result from moderate amine oxidation or low bioavailability of the reactive metabolites. In addition, with nitroarenes nitroso-intermediates are likely to be formed by reduction of a nitro group rather than the reoxidation of an amino group after complete reduction. Second, addition of a methyl group lowers adduct formation, nitro substituents in *para*-position to the methyl group of toluene derivatives are preferably reduced. Third, alternate pathways such as *C*-oxidation, ring hydroxylation, and possibly quinone–imine generation also may be important metabolic pathways. Production of nonhydrolyzable adducts or more rapid elimination of detoxification products could be the reason for unexpected low hemoglobin-binding indices in some cases.

The compounds 2,4,6-trinitrotoluene, 2,4- and 2,6-dinitrotoluene, 2-amino-4-nitrotoluene, and 2,4-diaminotoluene are classified as carcinogenic in experimental animals. 1,3-Diaminobenzene and 1,3-dinitrobenzene are suspected of having carcinogenic potential (*2*). This group of substances is therefore considered hazardous for exposed populations.

Soil in certain areas of former explosives production may contain rather large amounts of nitroarenes (*1*). The actual uptake by different possible routes by people living or working or both in these areas is difficult to assess but should be measured as a basis for risk assessment. We propose to use the major hemoglobin adducts of 2,4,6-trinitrotoluene, 2,6-dinitrotoluene, and 1,3-dinitrobenzene as dosimeters for exposure in biomonitoring of potentially exposed populations.

Simple correlations between hemoglobin binding and carcinogenicity of this group of compounds could not be detected. More information is needed to understand better the complex pharmacokinetics of these chemicals and eventually to proceed from exposure control to risk assessment.

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